Potential Tumor Suppressive Pathway Involving DUSP6/MKP-3 in Pancreatic Cancer

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We previously found frequent loss of heterozygosity at 12q21 and 12q22-q23.1 in primary pancreatic cancers, and the *DUSP6/MKP-3* gene residing in this region at 12q22 lost its expression in the great majority of pancreatic cancer cell lines. The *DUSP6/MKP-3* protein is a dual-specificity phosphatase that dephosphorylates the active form of ERK, making a feedback loop to control ERK activity. Gain-of-function mutations of *KRAS2* occur in the great majority of pancreatic cancer cells, and loss of expression of *DUSP6/MKP-3* may synergistically promote constitutive activation of ERK and uncontrolled cell growth. To study loss of the feedback pathway and its impact on pancreatic cancer cell growth, we first investigated the expression of *DUSP6/MKP-3* in primary pancreatic cancer tissues immunohistochemically; we found up-regulation in mildly as well as severely dysplastic/*in situ* carcinoma cells and down-regulation in invasive carcinoma, especially in the poorly differentiated type. Adenovirus-mediated reintroduction of *DUSP6/MKP-3* into cultured pancreatic cancer cells induced strong expression of recombinant *DUSP6/MKP-3* and reduction of phosphorylated ERK in a dose-dependent manner based on the multiplicity of infection and resulted in suppression of cell growth. Moreover, analyses by flow cytometry and immunocytochemistry revealed that the exogenous expression of *DUSP6/MKP-3* induced apoptosis. These results show that *DUSP6* exerts apparent tumor-suppressive effects *in vitro* and suggest that *DUSP6* is a strong candidate tumor suppressor gene at 12q22 locus. (Am J Pathol 2003, 162:1807-1815)

Chromosome 12q is one of the frequently hemizygously deleted chromosome arms in primary pancreatic cancer tissues.^{1,2} By detailed allelotyping analysis, we have detected regions of high incidence of loss of heterozygosity (LOH) at 12q21 and 12q22-q23.1.³ Moreover, LOH at 12q correlates with the poor prognoses typical of patients suffering from pancreatic cancer.⁴ In our quest for tumor suppressor genes in these regions, we found that expres-

sion of *DUSP6/MKP-3* (*DUSP6*) residing at 12q22 was lost in the great majority of cultured pancreatic cancer cells.⁵ *DUSP6* encodes a dual-specificity phosphatase specific for extracellular signal-regulated kinase (ERK).^{6,7} ERK is a key effector mitogen-activated protein kinase (MAPK) involved in the RAS-GTP signal transduction pathway.⁸ Active RAS-GTP mediated by signals from growth factor-receptor tyrosine kinase recruits and stimulates RAF-1, which leads to activation of MAPK/ERK kinase (MEK) and ERK by subsequent phosphorylation. Activated ERK translocates into the nucleus and activates various transcription factors, resulting in cell growth and differentiation.⁸ *DUSP6* can dephosphorylate activated ERK and shut down the growth-stimulating signals; this is thought to be one of the feedback loops for controlling MAPK signal pathways in normal cell growth.^{9,10} The very strict specificity for ERK and localization in the cytosol of *DUSP6* are unusual characteristics that differ greatly from those of other MAPK phosphatases such as MKP-1 and MKP-2.^{7,9} The localization in the cytoplasm of *DUSP6* makes it a more effective inactivator for ERK by retaining it in the cytoplasm and preventing it from translocation into the nucleus where the target effectors reside.¹¹ Most pancreatic cancer cells harbor the gain-of-function mutations in the *KRAS2* gene.^{12,13} The constitutively active RAS may presumably stimulate ERK through RAF and MEK, eventually resulting in uncontrolled cell growth. Inactivation of *DUSP6* may abrogate the feedback loop and contribute to uncontrolled cell growth, which in turn can lead to carcinogenesis and progression. Based on these perceptions, we hypothesized that *DUSP6* had a tumor suppressive function enrolled in pancreatic carcinogenesis and progression. To test this hypothesis, we investigated the expression of *DUSP6* in primary pancreatic cancer tissues and cultured pancreatic cancer cells and analyzed the effects of adenovirus-mediated exogenous expression of *DUSP6* in the cultured pancreatic cancer cells. We find down-regulation of *DUSP6* in cells of invasive carcinoma but

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up-regulation in precursor lesions, including mildly as well as severely dysplastic/*in situ* carcinoma cells in pancreatic tissues. Some cultured pancreatic cancer cells show hyperactivated ERK and abolishment of expression of DUSP6. Further, the adenovirus-mediated transfer of DUSP6 induces suppression of growth and apoptosis in the cultured pancreatic cancer cells.

Materials and Methods

Pancreatic Tissues

A total of 46 human pancreatic tissues (36 surgically resected and 10 autopsied) were available for this study. All of the surgically resected tissues were retrieved from the files of the Department of Gastroenterological Surgery, Tohoku University Hospital. They included 26 well or moderately differentiated ductal adenocarcinomas, 9 poorly differentiated adenocarcinomas and 1 adenocarcinoma. The autopsied tissues were retrieved from the files of the Department of Pathology, Tohoku University Hospital. They were from adults without known pancreatic disease, all fixed within 4 hours postmortem and with minimal autolytic changes. The use of clinical patients' tissues in this study was approved by the Ethical Committee of the Tohoku University School of Medicine. All of the tissues were fixed with 10% buffered formalin solution and embedded in paraffin. Two consecutive sections, 4 μ m in thickness, were prepared; one section was stained with hematoxylin and eosin, and the other was used for immunohistochemical studies.

Immunohistochemical Staining of DUSP6 in Primary Pancreatic Cancer Tissues

After deparaffinization and hydration, the sections were incubated with phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide for 30 minutes to block the endogenous peroxidase activity. The sections were subsequently immunostained by the indirect peroxidase method using the goat polyclonal anti-MKP-3 (C-20) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) specific for DUSP6 at 1:100 dilution with PBS containing 10% normal rabbit serum for the primary antibody reaction, the anti-goat IgG (H+L) antibody (Vector Laboratories Inc., Burlingame, CA) for the secondary antibody reaction, and streptavidine solution (Nichirei, Tokyo, Japan) for the streptavidine biotin reaction. The 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen, and hematoxylin was used for counterstaining. Specificity of the anti-MKP-3 antibody was tested by the peptide-neutralizing method using MKP-3 C-20 peptide (Santa Cruz Biotechnology, Inc.) as described in the manufacturer's instructions.

Pancreatic Cancer Cell Lines

Human pancreatic cancer cell lines, PK-1, PK-8, and PCI-35 were obtained by personal communications with

their original developers.^{14,15} SU.86.86, MIA PaCa-2, and BxPC-3 were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (Trace Scientific Ltd., Melbourne, Australia).

Immunoblot Analysis

Immunoblotting was performed basically as described previously¹⁶ using 10 to 20% polyacrylamide gradient gel (Bio-Rad, Hercules, CA) and Tris/glycine/sodium dodecyl sulfate (SDS) buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS, pH 8.3). Antibodies used were anti-MKP-3 polyclonal antibody (C-20) (Santa Cruz Biotechnology, Inc.) specific for DUSP6, anti-active/phosphorylated form of ERK-1 and 2 monoclonal antibody (Sigma, St. Louis, MO), anti-ERK1 monoclonal antibody (BD Biosciences, San Jose, CA), anti-ERK2 monoclonal antibody (BD Biosciences), anti-V5 monoclonal antibody (Invitrogen), anti- β -actin monoclonal antibody (Sigma), and horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin antibody (Amersham Biosciences, Inc., Little Chalfont, Buckinghamshire, UK). Blocking conditions and concentrations of antibodies followed the manufacturer's instructions. Signals were visualized by reaction with ECL Detection Reagent (Amersham Biosciences, Inc.) and digitally processed using LAS 100 Plus with Science Lab 99 Image Gauge (Fuji Photo Film Co. Ltd., Minamiashi-gara, Kanagawa, Japan).

Generation of the Replication-Deficient Adenoviral Vector Expressing DUSP6

The whole coding region of DUSP6 was amplified by polymerase chain reaction (PCR) with a primer set FULL (5'-TTCGGATCCGACCCCCATGATAGATACG-3' and 5'-CTACTCGAGCGTAGATTGCAGAGAGTCC-3') using a pooled cDNA prepared from a human liver cDNA library (Stratagene, La Jolla, CA) as the template. PCR was performed for 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute in GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The amplified cDNA was sequentially cloned into vectors of pcDNA3.1/V5-His, pCAcc, and pAdex1cw. The former was purchased from Invitrogen, and the latter two were obtained from RIKEN Bioresource Center (Tsukuba, Japan). A replication deficient adenovirus, AdDUSP6, was generated by using an Adenovirus Expression Vector Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions based on the method described by Miyake et al.¹⁷ A control vector containing the *lacZ* gene (AdLacZ) was prepared in the same way from pAdexcw-LacZ provided in the kit.

Adenovirus-Mediated DUSP6 Transfer

Monolayer cultures of the pancreatic cancer cells in 100-mm dishes at 70% confluence were infected at dif-

ferent multiplicities of infection (MOI) as described elsewhere.¹⁸ Every 24 hours until 72 hours after infection, both floating and adherent cells were collected and processed for immunoblotting. The immunoblotting was done as described above.

MTT Assay

Ten thousand cells in 50 μ l of the appropriate culture medium containing enough AdDUSP6 or AdLacZ to infect at MOI 10 and 50 were seeded into 96-well plates. The mock reaction contained no virus. Every 24 hours, the medium was replaced with 100 μ l of 0.05% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)/PBS (–) and then incubated for 1 hour. After the incubation, the MTT solution was removed by aspiration, and the cells were suspended in 100% ethanol. Absorption was measured at 590 nm.

Flow Cytometry

The cells were infected at MOI 50 with either AdDUSP6 or AdLacZ and collected 3 days later. “Mock” indicates an experiment without infection. The cells were washed with PBS (–) and then fixed overnight with 70% ethanol at –20°C. The fixed cells were pelleted, resuspended in 100 μ l of hypotonic citric buffer (192 mmol/L Na₂HPO₄, 4 mmol/L citric acid), and incubated for 20 minutes at room temperature. Then the cells were pelleted and suspended in PI/RNase/PBS (100 μ g/ml propidium iodide, 10 μ g/ml RNase A). FACS Calibur System (BD Immunocytometry Systems) was used for analysis of DNA content.

Immunocytochemistry

The cells were infected at MOI 50 with AdDUSP6. Three days later, both the floating and adherent infected cells were collected and fixed with 4% paraformaldehyde/PBS overnight at 4°C. The cells were washed with PBS and then spread on MAS-coated glass slides (Matsunami Glass Industry Ltd., Tokyo, Japan). The primary antibody reaction was accomplished by incubation with anti-V5 antibody diluted at 1:1000 in 10% goat serum/PBS at 4°C for 16 hours. The secondary antibody reaction was achieved by incubation with FITC-conjugated goat anti-mouse IgG (H+L) antibody (Zymed, San Francisco, CA) diluted at 1:40 into 10% goat serum/PBS for 1 hour at room temperature. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). The fluorescent image was visualized digitally by the fluorescence microscope system described previously.¹⁹

Statistical Analysis

Statistical differences in this study were analyzed using χ^2 test for the immunohistochemical study, Fisher's exact test for the immunoblotting analysis, and unpaired *t*-test

for MTT assay, performed by Statview 5.0 software (SAS institute Inc., Cary, NC).

Results

To determine a possible role for DUSP6 in pancreatic carcinogenesis and progression, we investigated the expression of DUSP6 in primary pancreatic cancer tissues by immunohistochemical methods. A total of 46 human pancreatic tissues were available for this study; of these, 36 tissues were obtained from surgically resected pancreata for primary invasive carcinoma and the remaining 10 tissues were obtained from autopsies of patients without known pancreatic disease. All of the tissues were fixed with 10% buffered formalin and embedded in paraffin. The indirect immunoperoxidase method was used for immunohistochemical staining of DUSP6. The antibody used was a polyclonal anti-DUSP6/MKP-3 (C-20) antibody raised in goat and purchased from Santa Cruz Biotechnology, Inc. Specificity of the antibody was tested by the peptide neutralization method performed according to the manufacturer's instructions. The immunoreactivity was completely abolished after the peptide neutralization in the immunohistochemical staining (compare Figure 1E with Figure 1A). The immunoreactivity for DUSP6 was evaluated in cells of invasive carcinoma and in the ductal epithelial cells, which were classified as normal, mild dysplasia, or severe dysplasia/carcinoma *in situ* by morphological findings based on the criteria as described previously.²⁰ The evaluation of immunoreactivity was graded and scored as negative in 0+, focally or weakly positive in 1+, consistently positive in 2+, and intensely positive in 3+, as shown in Figure 1. Results of the immunohistochemistry are summarized in Table 1. In 46 pancreata, 42 tissues contained ducts with normal epithelial cells, which showed varying immunoreactivity: 0+ or 1+ in 28 pancreata, and 2+ or 3+ in 14 pancreata. Ductal cells with mild dysplasia or severe dysplasia/carcinoma *in situ* were found in 25 and 12 of 46 pancreata, respectively. Of the 25 mildly dysplastic lesions, 7 were retrieved from autopsied pancreata. All of the severely dysplastic lesions were retrieved from pancreata with carcinoma. These dysplastic cells showed positive immunoreactivity except for one tissue and the majority of them, 23 of 25 mildly dysplastic areas and 11 of 12 severely dysplastic areas, showed 2+ or 3+ immunoreactivity. No difference in immunoreactivity was observed in mildly dysplastic lesions between autopsied samples and surgically resected samples. In pancreata with carcinoma, 34 tissues contained components of well or moderately differentiated ductal adenocarcinoma and 10 tissues contained components of poorly differentiated adenocarcinoma. In contrast to dysplastic cells, the immunoreactivity for DUSP6 was negative or weak/focal in the majority of the carcinoma tissues; 25 of 34 well or moderately differentiated adenocarcinomas and 9 of 10 poorly differentiated adenocarcinomas showed 0+ and 1+ reactivity. There was a significant correlation of high scores in dysplastic lesions and low scores in carcinoma lesions ($p < 0.001$ by χ^2 test). From these observations,

Figure 1. Immunohistochemical staining of DUSP6 in pancreatic tissues using the indirect peroxidase method and using DAB as a chromogen. Counterstained with hematoxylin. The immunoreactivity was evaluated with grading and scoring intensely positive as 3+ (**A**), consistently positive as 2+ (**B**), focally or weakly positive as 1+ (**C**), or negative as 0+ (**D**). In cells of M, mild dysplasia; S, severe dysplasia/carcinoma *in situ*; W/M, well or moderately differentiated ductal adenocarcinoma; P, poorly differentiated adenocarcinoma. N denotes normal ducts. **E** shows complete abolishment of the immunoreactivity by neutralization of the antibody.

we concluded that expression of DUSP6 was up-regulated in the majority of mildly dysplastic as well as severely dysplastic/*in situ* carcinoma cells but down-regulated in invasive carcinoma cells, especially in the poorly differentiated type.

To investigate the relationship between endogenous expression of DUSP6 and activation of ERK under either

wild-type or mutated *KRAS2*, we analyzed the expression of DUSP6 and active ERK in several pancreatic cancer cell lines. Pancreatic cancer cells growing exponentially in the culture medium containing 10% fetal bovine serum were collected at 70% confluence by scraping. The cells were lysed and processed for immunoblotting. As shown in Figure 2A, endogenous expression of DUSP6 was

Table 1. Immunohistochemical Staining of DUSP6 in Pancreatic Cancer Tissues

Histological type	IHC score				Total
	0+	1+	2+	3+	
Normal duct	16	12	11	3	42
Mild dysplasia	0	2	14	9	25
Severe dysplasia/CIS	1	0	6	5	12
Wel/Mod carcinoma	7	18	9	0	34
Por carcinoma	8	1*	1	0	10

*One case involved adenosquamous carcinoma.

Indicated are numbers and categories of lesions retrieved from 46 pancreata. IHC score 0+, no staining; 1+, weak/focal staining; 2+, consistent staining; 3+, intense staining as shown in Figure 1. CIS, carcinoma *in situ*; Wel/Mod, well or moderately differentiated ductal adenocarcinoma; Por, poorly differentiated adenocarcinoma.

detected only in PK-8 among the tested cell lines. More interestingly, the active/phosphorylated form of ERK (P-ERK) was detected in all of the tested cell lines except PK-8. All of the tested cells except BxPC-3 harbor oncogenic gain-of-function mutations of *KRAS2*, as we reported previously.²¹ We also investigated the expression of MKP-1 and MKP-2, both of which can inactivate the active form of ERK. We found that the expression of MKP-2 was relatively weak and that there was little expression of MKP-1 in any of the tested cell lines. These observations may suggest that constitutive activation of ERK depends on endogenous expression of DUSP6, although the result was not statistically significant ($p = 0.1667$ by Fisher's exact test). The constitutive activation may be partly the result of synergistically reduced or lost expression of MKP-1 and MKP-2 in some of these pancreatic cancer cell lines *in vitro*.

To determine the effect of expression of DUSP6 on pancreatic cancer cell growth, we constructed a replication deficient adenoviral vector harboring human *DUSP6* and studied its effect in the cultured pancreatic cancer cells. The adenoviral vector expressing DUSP6 with a V5-His tag in the carboxyl terminus (AdDUSP6) was constructed as shown in Figure 2B. The virus was generated at 1 to 5×10^9 pfu/ml in titer. We tested infection by the virus and analyzed its effects in several pancreatic cancer cell lines. Results for PCI-35 and PK-8 are presented as the representative phenotypes. The mutations of *KRAS2* in these cell lines were previously reported as G12R in PK-8 and G12D in PCI-35. The wild-type *KRAS2* allele was deleted in PK-8.²¹ Efficacy of infection of a replication-deficient adenoviral vector in these pancreatic cancer cells was assessed by infection of the control adenoviral vector expressing β -galactosidase, AdLacZ. The *in situ* β -galactosidase activity was detected efficiently in a dose-dependent manner based on multiplicity of infection (MOI). Infection at MOI 50 induced the activity in approximately 90% of the cells in both cell lines (data not shown). The cells were infected either with AdDUSP6 or AdLacZ at MOI 10 and 50, and then expression of the recombinant DUSP6-V5 was analyzed 2 days later by immunoblotting using an antibody specific for the V5 tag. As shown in Figure 2C, infection with AdDUSP6 induced strong expression of the recombinant DUSP6 in a MOI dependent manner. The expression was well beyond the

Figure 2. A: Expression of ERKs and MKPs in cultured pancreatic cancer cells. The pancreatic cancer cells growing exponentially in culture media containing 10% fetal bovine serum were collected at 70% confluence by scraping. The cells were lysed and processed for immunoblotting performed with antibodies to active/phosphorylated form of ERK (p-ERK), ERK2, ERK1, DUSP6, MKP-1, MKP-2, and β -actin. Lane 1, PK-8; lane 2, PCI-35; lane 3, PK-1; lane 4, SU.86.86; lane 5, MIA PaCa-2; lane 6, BxPC-3. B: Structure of the replication-deficient adenoviral vector expressing DUSP6-V5-His. The entire coding region of *DUSP6* joined with V5 and His tags in its carboxyl terminus was cloned under cytomegalovirus enhancer (CMV) and chicken β -actin promoter (CA) in the E1 region of an Ad5 fragment which lacked E3. C: AdDUSP6 infection resulted in strong expression of the recombinant DUSP6 and suppression of active ERK in a dose-dependent manner based on multiplicity of infection (MOI). Pancreatic cancer cell lines, PCI-35 and PK-8 were infected at different MOI either with AdDUSP6 and AdLacZ. "Mock" designates no virus infection. Forty-eight hours after the infection, both floating and adherent cells were collected, processed for whole-cell lysate, and assayed by immunoblots with antibodies to V5 tag, DUSP6, active/phosphorylated form of ERK (p-ERK), ERK2, ERK1, and β -actin. Lane 1, mock infection; 2, AdDUSP6 at MOI 10; 3, AdDUSP6 at MOI 50; 4, AdLacZ at MOI 10; 5, AdLacZ at MOI 50.

endogenous expression level, 2.0- and 4.6-times increased in MOI 10 and 50, respectively, comparing with mock in PK-8 noted by measuring densities in the blot probed with anti-DUSP6 antibody. The blot was probed

Figure 3. Growth of cells as shown by MTT assay. Ten thousand cells in 50 μ l of the culture medium containing AdDUSP6 or AdLacZ to infect at MOI 10 and 50 were seeded in 96-well plates. The mock reaction contained no virus. On each day, cells were incubated with 0.05% MTT/PBS (-) for 1 hour and then suspended in 100% ethanol. Absorption at 590 nm was measured. Mean $\pm$ SD of actual values of the measurement from 8 independent wells in each experimental group were plotted on a logarithmic scale. Statistical analysis was performed by unpaired Student's *t*-test.

Figure 4. Increase in sub-G1 fraction after infection with AdDUSP6. The cells were infected at MOI 50 with either AdDUSP6 or AdLacZ and collected 3 days later. "Mock" indicates an experiment without infection. The cells were fixed overnight with 70% ethanol at -20°C . The fixed cells were stained with 100 $\mu\text{g}/\text{ml}$ propidium iodide in PBS containing 10 $\mu\text{g}/\text{ml}$ RNase A after hypotonic treatment with citric buffer. FACS Calibur System (BD Immunocytometry Systems) was used for analysis of DNA content. Bars and numbers indicate the range of the sub-G1 fraction and its rate in total counts, respectively.

with anti-active/phosphorylated form of ERK (p-ERK) antibody to examine the specific effects of the adenovirus-induced expression of DUSP6 on ERK activity. As shown in Figure 2C, AdDUSP6 infection induced marked reduction of p-ERK in a MOI-dependent manner in PCI-35, indicating that recombinant DUSP6 was overexpressed and specifically dephosphorylated ERK. In PK-8, little expression of p-ERK was seen in either the mock or the infected cells. We analyzed the expression of recombi-

nant DUSP6 for 3 days after infection; it could be detected 24 hours through 72 hours after infection, and reached its highest level at 48 hours after infection (data not shown).

Next we analyzed whether the growth of pancreatic cancer cells was affected by exogenous expression of DUSP6 mediated by the adenoviral infection. Ten thousand cells were infected either with AdDUSP6 or AdLacZ at MOI 10 and 50 and monitored for 4 days to observe the effects of infection on logarithmic growth by MTT assay. As shown in Figure 3, infection with AdDUSP6 induced suppression of growth strongly in PCI-35 and moderately in PK-8 in a MOI-dependent manner with causing some remarkable decrease in the number of viable cells, indicating the cytotoxic effects of AdDUSP6.

To elucidate the mechanisms of growth suppression, we analyzed the cell cycle status of infected cells by flow cytometry. The cells were infected at MOI 50 with either AdDUSP6 or AdLacZ, collected 3 days later, and processed for analyzing DNA content by treatment with hypotonic citric buffer and staining with propidium iodide. As shown in Figure 4, an increase in the sub-G1 fraction after infection with AdDUSP6 was observed in both cells as compared with mock cells or those infected with AdLacZ. Because the sub-G1 fraction mainly represents apoptotic cells,²² the data strongly suggest that AdDUSP6 infection induced cells to apoptosis. The degree of the sub-G1 fraction was compatible with the suppressive effect on cell growth, ie, nearly a ten-fold increase compared to mock or AdLacZ infection in PCI-35, which showed a marked reduction of cell growth. The three-fold increase in PK-8 showed a lesser degree of reduction.

To determine whether the exogenous expression of DUSP6 was directly related to the apoptosis of cells, infected cells with AdDUSP6 at MOI 50, both floating and adherent, were collected 72 hours after infection, fixed, and spread on coated glass slides. The recombinant DUSP6 was visualized by the indirect immunocytochemical method using anti-V5 antibody. As shown in Figure 5, expression of the recombinant DUSP6 was observed in apoptotic cells that typically showed fragmentation of their nuclei and shrinkage of cellular shapes. Cells with deformed nuclei, which were apparently in a preapoptotic state, also expressed the exogenous DUSP6. Therefore, the recombinant DUSP6 expression was related directly to the apoptosis of the infected cells.

Discussion

As demonstrated, we found that the expression of DUSP6 was up-regulated in a majority of dysplastic cells in pancreatic ducts but down-regulated in invasive carcinoma cells, especially in poorly differentiated ones, in pancreatic tissues. Endogenous expression of DUSP6 seemed to be inversely correlated with constitutive activation of ERK in cultured pancreatic cancer cells. Moreover, the adenovirus-mediated transfer of DUSP6 into cultured pancreatic cancer cells resulted in suppression of growth and induction of apoptosis, although the expression level

Figure 5. The recombinant DUSP6-V5 was detected directly in apoptotic cells by immunocytochemical staining. Three days after infection with AddUSP6 at MOI 50, both floating and adherent cells were collected, fixed with 4% paraformaldehyde/PBS, and then spread on coated glass slides. Indirect immunofluorescence staining was performed using the anti-V5 antibody and FITC-conjugated secondary antibody. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Non-specific cytoplasmic fluorescence is shown in red. Images represented are nuclei (**A**, **E** and **I**), V5-tag staining (**B**, **F** and **J**), cytoplasm (**C**, **G** and **K**), and those overlayed (**D**, **H** and **L**). Note apoptotic cells with fragmented nuclei in shrunken cytoplasm (**arrow**) and a cell harboring a deformed nucleus (**asterisk**) and expressing the recombinant DUSP6 (shown as green in V5 tag-staining).

of the adenovirus-mediated transfection was much higher than that under physiological conditions.

It is well established that dysplastic cells in pancreatic ducts correspond to precancerous lesions or early neoplastic changes.²³ Molecular studies indicate that an oncogenic gain-of-function mutation of *KRAS2* is frequently found not only in invasive carcinomas but also in the dysplastic cells of the pancreas.^{24–26} The mutated RAS can activate RAF and downstream MAP kinase cascades including ERK. Activated ERK translocates into the nucleus and induces various gene expressions.⁹ Among the early genes that ERK induces are genes encoding MAP kinase phosphatases (MKPs), including *DUSP6*, that dephosphorylate and inactivate ERK itself, thus forming a negative feedback loop.^{6,10} Taken in consideration together with these observations, our immunohistochemical findings suggest that up-regulation of *DUSP6* in the pancreatic ductal dysplastic cells may be a result of accelerating the negative feedback loop for activated ERK induced by the oncogenic mutation of *KRAS2* occurring in the dysplastic cells. However, down-regulation of *DUSP6* in invasive carcinoma cells may result in loss of this loop and constitutive activation of ERK, which can

lead to uncontrolled cell growth and promotion of malignant phenotypes. This model is supported by findings of expression of the active/phosphorylated form of ERK and loss of endogenous expression of *DUSP6* in a majority of pancreatic cancer cell lines. Interestingly, endogenous expression of *DUSP6* was observed in one cell line (PK-8) among those we tested, and active ERK was detected in all of the cell lines except for the *DUSP6*-maintained PK-8. ERK can be a substrate for MKP-1 and MKP-2 as well as *DUSP6*, although the substrate specificity differs widely among the MKPs.⁹ We investigated the expression of MKP-1 and MKP-2 to determine whether specific activation of ERK correlates with those MKPs. Loss of expression of MKP-1 and reduced expression of MKP-2 were seen in all of the tested cells, appearing not to correlate directly with the active status of ERK. These data suggest that constitutive activation of ERK may depend on endogenous loss of expression of *DUSP6* rather than on that of MKP-1 or MKP-2.

If the constitutively active ERK induced by loss of function of *DUSP6* directly promote growth of pancreatic cancer cells, recovery of expression of *DUSP6* should inactivate ERK and stop or retard the cell growth. To

investigate this perception, we constructed an adenoviral vector harboring *DUSP6* for efficient introduction and tested its effect. We observed that exogenous expression of *DUSP6* dephosphorylated ERK specifically and induced suppression of cell growth although the expression level was much higher than the physiological level. In cells tested in this study, PCI-35 revealed remarkable suppression of growth after Ad*DUSP6* infection, but the effect was less remarkable in PK-8. PK-8 expressed *DUSP6* endogenously and active ERK very weakly, if at all; it is possible that the exogenous *DUSP6* may be able to work less specifically. These results suggest that the growth of pancreatic cancer cells lacking endogenous expression of *DUSP6* may depend on ERK activity more strongly than that of cells maintaining their endogenous expression; therefore, the former type of cell, like PCI-35, is more sensitive to exogenous expression of *DUSP6*.

Our results also suggest that down-regulation of *DUSP6* protein levels would cause an increase in phosphorylated ERK levels, especially under the mutated *KRAS2*. Experimental knockout of *DUSP6* under activation of RAS should be further investigated.

The adenovirus-mediated exogenous expression of *DUSP6* induced apoptosis in cultured pancreatic cancer cells *in vitro*, indicating that suppression of ERK may provoke an apoptotic pathway. The link between apoptosis of cells and suppression of ERK has been reported by showing anti-apoptotic function of up-regulated ERK^{27–29} or induction of apoptosis by suppression of ERK.³⁰ Direct evidence of the involvement of *DUSP6* in the pro-apoptotic pathway has recently been provided by the data showing that down-regulation of *DUSP6* by nitric oxide mediates up-regulation of ERK and survival of endothelial cells by means of anti-apoptosis.³¹ All of these reports indicate that suppression of ERK by *DUSP6* may induce apoptosis, which is consistent with our result. Another possible mechanism has been shown by Dimmeler et al,³² who indicated that degradation of antiapoptotic effector BCL-2 is promoted by expression of *DUSP6* in human umbilical endothelial cells. The detailed mechanism of the induction of apoptosis by exogenous expression of *DUSP6* in the pancreatic cancer cells should be clarified by further investigations of the MAPK signaling pathway as well as the apoptotic pathway.

The underexpression of *DUSP6* and misregulation of p-ERK has relevance for therapeutics, since downstream MAPKs are not usually targeted (RAS usually is). The current work suggests that the efficient targeting of RAS proteins would leave cells with a residual misregulation of downstream effectors, which should be taken into account in the design and testing of novel therapeutics.

In this study, suppression of cell growth and induction of apoptosis were observed after exogenous expression of *DUSP6* in pancreatic cancer cells. These results point to *DUSP6* as one of the key players in the tumor suppressive pathway and as a promising molecular target for curing patients with pancreatic cancer.

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